

Engineering of *Corynebacterium glutamicum* with a NADPH-generating Glycolytic Pathway for L-Lysine Production

Running title: *C. glutamicum* with a NADPH-generating Glycolytic Pathway

Seiki Takeno,¹ Ryosuke Murata,¹ Ryosuke Kobayashi,¹ Satoshi Mitsuhashi,² and Masato Ikeda^{1*}

*Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, Nagano 399-4598, Japan,*¹ *and Bioprocess Development Center, Kyowa Hakko Bio Co., Ltd., Tsukuba, Ibaraki 305-0841, Japan*²

*Corresponding author. Mailing address: Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, Nagano 399-4598, Japan. Phone: +81-265-77-1614. Fax: +81-265-77-1629. E.mail: m_ikeda@shinshu-u.ac.jp.

ABSTRACT

A sufficient supply of NADPH is a critical factor in L-lysine production by *Corynebacterium glutamicum*. Endogenous NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) of *C. glutamicum* was replaced with nonphosphorylating NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (GapN) of *Streptococcus mutans*, which catalyzes the reaction of glyceraldehyde 3-phosphate to 3-phosphoglycerate with the reduction of NADP⁺ to NADPH, resulting in the reconstruction of the functional glycolytic pathway. Although the growth of the engineered strain on glucose was significantly retarded, a suppressor mutant with an increased ability to utilize sugars was spontaneously isolated from the engineered strain. The suppressor mutant was characterized by the properties of the GapN as well as the nucleotide sequence of the gene, confirming that no change occurred in either the activity or basic properties of GapN. The suppressor mutant was engineered into an L-lysine-producing strain by plasmid-mediated expression of the desensitized *lysC* gene, and the performance of the mutant as an L-lysine producer was evaluated. The amounts of L-lysine produced by the suppressor mutant were higher than those produced by the reference strain, which was created by replacement of the preexisting *gapN* gene in the suppressor mutant with the original *gapA* gene, by ~70% on glucose, ~120% on fructose, and ~100% on sucrose, indicating that the increased L-lysine production was attributed to the GapN. These results demonstrate effective L-lysine production by *C. glutamicum* with an additional source of NADPH during glycolysis.

INTRODUCTION

L-Lysine has been manufactured by the fermentation method using mutants of *Corynebacterium glutamicum*; presently, more than 750,000 tons of L-lysine are produced by this method per year (28). For efficient production of L-lysine, the supply of cofactor NADPH is one of the critical factors, because 1 mol of L-lysine requires 4 mol of NADPH for its biosynthesis from glucose in *C. glutamicum* (23). In this organism, it has been reported that NADPH is generated mainly through an oxidative pentose phosphate pathway (PPP), and partly by NADP-dependent isocitrate dehydrogenase and NADP-dependent malic enzyme (17, 18, 19). The importance of the PPP for L-lysine production is particularly obvious when fructose is used as a carbon source: only 14.4% of carbon is channeled through the PPP when fructose is used, whereas 62.3% is channeled through the PPP when glucose is used (14).

Becker et al. reported that overexpression of the homologous *fbp* gene encoding fructose 1,6-bisphosphatase leads to a redirection of carbon from glycolysis

toward the PPP, resulting in a significant improvement of L-lysine production on fructose, as well as on glucose and sucrose (3). Similarly, strategies including disruption of the phosphoglucose isomerase gene (20), overexpression of the glucose 6-phosphate dehydrogenase gene (2), and introduction of a mutant allele of the 6-phosphogluconate dehydrogenase gene encoding an enzyme less-sensitive to feedback inhibition (25) lead to improved L-lysine production in *C. glutamicum*. Furthermore, Kabus et al. have demonstrated that the heterologous expression of *pntAB* genes, which encode a membrane-bound transhydrogenase that catalyzes the reduction of NADP^+ to NADPH by the oxidation of NADH to NAD^+ , effectively increases the production of L-lysine by *C. glutamicum* in a manner independent of sugar metabolism (13).

Regardless of the flux distribution between glycolysis and the PPP, all carbon from glucose, fructose, and sucrose passes through the step of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which catalyzes the reaction of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate with the reduction of NAD^+ to NADH. *C. glutamicum* has two GAPDHs: GapA, an NAD-dependent GAPDH, and GapB, which represents a dual cofactor signature with a preference for NADP^+ (7, 11). Transcription of the two *gap* genes has been shown to be inversely regulated between cells grown on glucose and acetate: in acetate, *gapB* expression was induced by several-fold, although that of *gapA* was repressed by about 2-fold (7). Furthermore, functional analysis has demonstrated that GapA contributes to both glycolysis and gluconeogenesis, whereas GapB is involved only in gluconeogenesis (27). If the NADPH-generating GapB could be altered to work for glycolysis, this engineering should improve L-lysine production. To address this, we attempted to isolate suppressor mutants from a *C. glutamicum* ΔgapA strain that cannot grow on glucose. However, unfortunately, we found that the resulting mutants were not sufficiently capable of growing on glucose to permit the efficient production of L-lysine. These results led us to anticipate difficulties in exploiting the endogenous *gapB* gene for glucose catabolism, probably due to an intracellular environment in which NAD^+ is more abundant than NADP^+ (15).

On the other hand, non-phosphorylating NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (GapN, EC. 1.2.1.9), which catalyzes the irreversible oxidation of glyceraldehyde 3-phosphate to 3-phosphoglycerate with the reduction of NADP^+ to NADPH, is found in photosynthetic eukaryotes (10, 21) and in some gram-positive bacteria, such as *Streptococcus* (1, 4, 6, 9) and *Clostridium* species (8). In this work, we attempted to use the heterologous *Streptococcus mutans gapN* gene to engineer *C. glutamicum*. Our objectives are: (i) to utilize the GapN enzyme as an additional source of NADPH during glycolysis, and (ii) to lead the engineered strain to

an effective L-lysine-producing strain.

MATERIALS AND METHODS

Bacterial strain, growth conditions, and plasmids. The wild-strain *C. glutamicum* ATCC 13032 was used in this study. Complete medium BY (32) and minimal medium MM (32) were used for the cultivation of ATCC 13032 and its derivative mutants. For the growth test, liquid culture using MM was done in L-type test tubes shaken on a Monod shaker at 48 strokes/min. LFG1 medium (24) was also used for growth test and for L-lysine production; it consisted of (per liter): sugar at the indicated concentration, 10 g of corn steep liquor, 45 g of $(\text{NH}_4)_2\text{SO}_4$, 2 g of urea, 0.5 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 mg of biotin, and 30 g of CaCO_3 (pH 7.0). The growth test using LFG1 medium was done at 30°C in test tubes reciprocally shaken at 120 strokes/min. Seed medium used for the L-lysine production was made by the addition of 2% glucose (w/v) and 1% CaCO_3 (w/v) at final concentration to the BY medium. *S. mutans* NBRC 13955 provided by the National Institute of Technology and Evaluation (Japan) was used for the isolation of the genomic DNA. The streptococcal cells were grown on the agar medium containing (per liter) 5 g of polypeptone, 5 g of yeast extract, 5 g of glucose, and 1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ at 37°C under anaerobic conditions. *Escherichia coli* DH5 α was used as a host for DNA manipulation. LB medium (30) was used to grow *E. coli*. Solid medium was made by the addition of Bacto-Agar (Difco) to a concentration of 1.6%. Plasmid pESB30, which is non-replicative in *C. glutamicum*, was used for gene disruption and gene replacement in *C. glutamicum* (22). Plasmid pCS299P carrying the kanamycin resistance gene (22) was used for the expression of the desensitized *lysC* gene. Plasmid pCAK311, which contains the desensitized *lysC* gene (*lysC*^{T311}) in vector pCS299P, was constructed to create L-lysine-producing strains as follows. The genomic region comprising the desensitized *lysC* gene from strain *C. glutamicum* AK-1, which was previously constructed as a defined L-lysine-producer (26), was amplified by PCR using two primers, reLysC311F3 and reLysC311R3, with the AK-1 genomic DNA as a template. The reaction was performed according to the following program: heating to 94°C for 5 min; 25 cycles each consisting of 94°C for 30 sec, 60°C for 30 sec; and 72°C for 2.2 min; followed by extension for 5 min. The resulting 2.0-kb fragment was digested with BamHI and then ligated to BamHI-digested pCS299P.

Recombinant DNA techniques. Standard protocols (30) were used for the construction, purification and analysis of plasmid DNA and for the transformation of *E. coli*.

Chromosomal DNA was extracted from *C. glutamicum* and *S. mutans* by the methods described by Saito and Miura (29) and by Schroeder et al. (31), respectively. Transformation of *C. glutamicum* by electroporation was carried out by the method described by van der Rest et al. (33) using the Gene Pulser II electroporation system (Bio-Rad Laboratories Inc., Hercules, CA, USA). PCR was performed using a DNA thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA, USA) using Pyrobest® DNA Polymerase (Takara Bio Inc., Shiga, Japan). Each of these programs will be described in greater detail in subsequent sections. Sequencing to confirm the nucleotide sequences of relevant DNA regions were performed using an ABI PRISM™ 377 DNA sequencer from Applied Biosystems, with ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer, USA). The subsequent electrophoresis analysis was carried out by Pageset SQC-5ALN 377 (Toyobo, Japan).

Strain construction. The sequences of the primers used in this study are listed in Table 1. All primers were designed based on the genomic sequences of *C. glutamicum* (BA000036) or *S. mutans* (AE014133), which are publicly available at http://gib.genes.nig.ac.jp/single/index.php?spid=Cglu_ATCC13032 and http://gib.genes.nig.ac.jp/single/index.php?spid=Smut_UA159, respectively.

For the chromosomal deletion of the *gapA* gene, plasmid pCΔ*gapA* containing the internally deleted *gapA* gene was constructed as follows. The 5'-region of the *gapA* gene was amplified by PCR using two primers, *gapAup5* and *gapAN3*, with the wild-type ATCC 13032 genomic DNA as a template. Similarly, the 3'-region of the gene was amplified using *gapAC5* and *gapAdn3*. Each of these reactions was performed according to the following program: heating to 94°C for 5 min; 25 cycles each consisting of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min; followed by extension for 3 min at 72°C, which amplified the 950-bp and the 850-bp fragments, respectively. As the two primers, *gapAN3* and *gapAC5*, were complementary to each other, fusion PCR was performed using the two resulting purified fragments as templates, and the primers *gapAup5* and *gapAdn3*, according to the following program: heating to 94°C for 5 min; 25 cycles each consisting of 94°C for 30 sec and 70°C for 2 min; followed by extension for 3 min at 70°C. The resulting 1.8-kb fragment contained the deleted *gapA* gene which was shortened by in-frame deletion of the inner sequence. The fragment was digested with BclII and then ligated to BamHI-digested pESB30 to yield pCΔ*gapA*. Defined chromosomal deletion of the *gapA* gene was accomplished using pCΔ*gapA* via two recombination events as described previously (26). The strain carrying the *gapA* gene deletion in a wild-type background was designated strain

Δ gapA.

For the chromosomal deletion of the *gapB* gene, plasmid pC Δ gapB containing the internally deleted *gapB* gene was constructed as follows. The 5'-region of the *gapB* gene was amplified by PCR using two primers, gapBup5 and gapBN3, with the wild-type ATCC 13032 genomic DNA as a template. Similarly, the 3'-region of the gene was amplified using gapBC5 and gapBdn3. Each of these reactions was performed according to the following program: heating to 94°C for 5 min; 25 cycles each consisting of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min; followed by extension for 3 min at 72°C, which amplified the 750-bp and the 850-bp fragments, respectively. As the two primers gapBN3 and gapBC5 were complementary to each other, fusion PCR was performed using the two resulting purified fragments as templates, and the primers gapBup5 and gapBdn3, according to the following program: heating to 94°C for 5 min; 25 cycles each consisting of 94°C for 30 sec and 70°C for 2 min; followed by extension for 3 min at 70°C. The resulting 1.6-kb fragment contained the deleted *gapB* gene which was shortened by in-frame deletion of the inner sequence. The fragment was digested with BclI and then ligated to BamHI-digested pESB30 to yield pC Δ gapB. Defined chromosomal deletion of the *gapB* gene was accomplished using pC Δ gapB via two recombination events as described previously (26). The strain carrying the *gapB* gene deletion in a wild-type background was designated strain Δ gapB. In a similar way, a *gapA* and *gapB* double mutant was created by the deletion of the *gapB* gene from strain Δ gapA, which was designated strain Δ gapAB.

For replacement of the *C. glutamicum* chromosomal *gapA* gene with the *S. mutans* *gapN* gene, plasmid pCSmgapN was constructed. The upstream and downstream regions of the *C. glutamicum* *gapA* gene open reading frame (for convenience, referred to as Fragments A and B, respectively) were amplified by pairs of primers, CGLgapAupXBF and gapNKob3, and gapNKob5 and CGLgapAdown900R, respectively, with the wild-type ATCC 13032 genomic DNA as a template. The *gapN* gene was amplified by PCR using two primers, gapNKobF and gapNkobR, with the *S. mutans* NBRC 13955 genomic DNA as a template. Each of the reactions was performed according to the following program: heating to 94°C for 5 min; 20 cycles each consisting of 94°C for 1 min, 54°C for 2 min, and 72°C for 2 min; followed by extension for 3 min at 72°C. Fragment A, *gapN* gene, and Fragment B were fused by stepwise procedures using PCR. The resulting 3.1-kb product was digested with BamHI, and then ligated to BamHI-digested pESB30 to yield pCSmgapN. Replacement of the internally deleted *gapA* gene with the intact *S. mutans* *gapN* gene in strain Δ gapAB was conducted using pCSmgapN via two recombination events as described previously (26).

The resulting strain was designated strain GPN.

For replacement of the *gapN* gene of the suppressor mutant RE2 with the *C. glutamicum gapA* gene, plasmid pCgapA was constructed. The genomic region comprising the *gapA* gene was amplified by PCR using two primers, CGLgapAup900F and CGLgapAdown900R, with the wild-type ATCC 13032 genomic DNA. The reaction was performed according to the following program: heating to 94°C for 5 min; 25 cycles each consisting of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1.5 min; followed by extension for 5 min at 72°C. The resulting 2.8-kb fragment was digested with BamHI, and then ligated to BamHI-digested pESB30 to yield pCgapA. Replacement of chromosomal *gapN* with *gapA* in strain RE2 was conducted using pCgapA via two recombination events as described previously (26). The resulting strain was designated strain RE2A.

Preparation of soluble fraction and enzyme assays. *C. glutamicum* strains were grown to late log phase in 200 mL of MM liquid medium containing 1% (w/v) glucose in 2-liter flasks reciprocally shaken at 120 strokes/min. Cells were collected by centrifugation at 10,000 x *g* for 10 min, then washed once with 50 mM triethanolamine hydrochloride (pH 8.0) containing 1 mM ethylenediaminetetraacetic acid and 1 mM dithiothreitol. The cells were suspended in 4-volumes of the same buffer, and sonicated on ice for 5 min using a ultrasonic disruptor UD-200 (Tomy Seiko Co. Ltd., Tokyo, Japan). Cell debris was removed by centrifugation at 10,000 x *g* for 30 min, and the supernatant was further ultracentrifuged at 100,000 x *g* for 60 min using an Optima™ TL Ultracentrifuge (Beckman Coulter Inc., Fullerton, CA, USA). The resulting supernatant was provided for the enzyme assay. All steps were done at 4°C unless otherwise stated. GAPDH activity was spectrophotometrically measured according to the method described by Omumasaba et al. (27) using a Shimadzu MultiSpec-1500 spectrophotometer (Shimadzu Co., Kyoto, Japan). Nonphosphorylating NADP-dependent GapN activity was measured according to the method described by Crow et al (6). One unit of activity is defined as 1 μmol of NAD(P)H formed per min.

L-Lysine production. *C. glutamicum* cells that had been cultured for one day on a BY plate were inoculated into 5 mL of the seed medium in test tubes and cultivated for 1 day with reciprocal shaking at 120 strokes/min at 30°C. After the glucose initially added into the seed medium was consumed, 0.5 mL of the seed culture was transferred into 5 mL of LFG1 medium containing 3% (w/v) glucose, fructose, or sucrose, then reciprocally shaken at 120 strokes/min at 30°C for 4 days.

Analysis. The bacterial growth was monitored by measuring OD₆₆₀ of the culture broth with a Miniphoto 518R spectrophotometer (Taitec, Saitama, Japan). Glucose concentration was determined according to the method described by Ohnishi et al (26). L-Lysine titer was determined as L-lysine HCl according to the method described by Ohnishi et al (26). Fructose and sucrose concentrations were determined using an F-kit Sucrose/D-Glucose/D-Fructose (Roche Diagnostics, Basel, Switzerland). Protein content was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories).

RESULTS

Roles of the two *C. glutamicum* GAPDHs in carbon metabolism. *C. glutamicum* has two GAPDHs, GapA and GapB. We obtained the corresponding gene-deletion mutants (strains Δ gapA and Δ gapB) from wild-type ATCC 13032, and compared their abilities to utilize carbon sources using a minimum medium MM (Fig. 1). Strain Δ gapA exhibited no growth on glucose and impaired growth on acetate. The *gapB* disruption appeared to have scarcely any effect irrespective of the carbon source tested. These results indicate that GapA is indispensable to glycolysis and also involved in gluconeogenesis, while GapB is responsible only for gluconeogenesis. These results agree with the findings obtained by Omumasaba et al. (27) with the use of another wild-type strain, *C. glutamicum* R. We also constructed strain Δ gapAB, a double-deleted mutant. This strain showed no growth on either glucose or acetate, confirming that no other GAPDH isozymes are present in this organism. All mutants showed comparable levels of growth on the complete medium (BY).

Expression of *S. mutans gapN* gene in *C. glutamicum*. We attempted to create a new step allowing NADPH supply in the glycolytic pathway of *C. glutamicum* using a GapN enzyme from *S. mutans*. For this purpose, the heterologous *gapN* gene was introduced into strain Δ gapAB so that the deleted *gapA* gene was replaced with the full-length *gapN* gene. This experimental setup should reveal the effects of GapN on sugar metabolism in a straightforward manner, because no intrinsic GAPDHs are present in strain Δ gapAB. The introduction of the *gapN* gene conferred the ability to grow on the MM with glucose on the double-deleted mutant (Fig. 2). On the other hand, the resulting strain GPN failed to grow on acetate (data not shown). These results indicate that this sugar catabolism occurred by a modified glycolytic pathway reconstructed by the irreversible GapN. A soluble fraction prepared from strain GPN grown on the same MM medium with glucose exhibited a comparably moderate level of NADP-dependent

GapN activity (Table 2). Taken together, we concluded that the engineered strain performed the glycolytic process *via* GapN accompanying the generation of NADPH. However, both a prolonged lag phase and lower growth rate compared to the wild-type and Δ gapB strains were observed in strain GPN (Fig. 2).

Isolation of spontaneous mutants with improved growth on glucose. Judging from the insufficient growth, as shown in Fig. 2, strain GPN is likely to be a less advantageous host for L-lysine production. Unfortunately, this disadvantage was more marked when the strain was grown on fermentation medium LFG1 containing 5% glucose for reasons not understood (Fig. 3). For the initial five days, strain GPN didn't show significant growth, like strain Δ gapAB which was unable to utilize glucose. However, after a long lag phase, explosive growth was observed in the culture, suggesting that suppressor mutants which had acquired an increased ability to grow on the glucose-based medium appeared spontaneously. Forty candidates were isolated from the broth at the end of the culture and classified into several groups according to their growth rates on glucose (data not shown). Among these, mutant RE2 showed the most improvement in growth rate on MM and especially on LFG1 medium (Fig. 2 and 3), and was therefore used for further analysis. No growth on acetate was observed in mutant RE2 (data not shown), which is reasonably explained by the irreversible reaction of the GapN enzyme. We also examined whether a similar fast-grower like strain RE2 appeared when an outgrowth culture of strain GPN was used to inoculate fresh MM medium, but no differences in its growth behavior were observed at least within three serial cultivations, suggesting that no suppressor mutant appears in MM medium for unknown reasons. On the other hand, such a suppressor mutant was ever obtained from strain Δ gapAB neither in LFG1 nor in MM media.

Mutant RE2 should contain some mutation(s) that confer increased ability to grow on sugar. Even if any such beneficial mutations for growth occurred, however, the ability to generate NADPH was a minimum requirement that had to be preserved in the GapN enzyme if we were to meet our objective. We assayed the GapN activity of strain RE2 and found that it had a level of NADP-dependent GapN activity similar to that of its parental strain GPN (Table 2). Moreover, we sequenced the *gapN* ORF of strain RE2 and also the regions covering the 500-bp upstream to the 300-bp downstream of the gene, revealing that no mutation was present in the regions that could affect the expression of the *gapN* gene or the enzymatic properties of GapN. Thus strain RE2 was a host strain that fulfilled our requirements.

L-Lysine production by strain RE2. We evaluated the performance of strain RE2 as a host for L-lysine production. For this purpose, plasmid pCAK311 (*lysC*^{T311I}) which could confer overproduction of L-lysine on a wild-type strain was introduced into strain RE2, its parental strain GPN, strain Δ gapB, and wild-type ATCC 13032. The resulting plasmid carriers were cultivated in LFG1 medium containing 3% glucose, fructose, or sucrose. The sugar initially added to the medium was completely consumed within 4 days in all samples except those using strain GPN. Only the plasmid carrier of strain GPN showed marginal growth with most sugar unconsumed under the conditions, and thus was excluded from the comparative analysis. As shown in Fig. 4, the concentrations of L-lysine produced by strain Δ gapB/pCAK311 were lower than those produced by strain ATCC 13032/pCAK311 by 26-30% on all carbon sources tested. Strain RE2/pCAK311 exhibited considerably increased L-lysine production compared to both strain ATCC 13032/pCAK311 and strain Δ gapB/pCAK311 on all sugars. On glucose, strain RE2/pCAK311 produced 1.4- and 1.7-fold more L-lysine (39.0 mM, 0.234 mol L-lysine /mol sugar) than strains ATCC 13032/pCAK311 (28.1 mM, 0.169 mol L-lysine /mol sugar) and Δ gapB/pCAK311 (23.4 mM, 0.140 mol L-lysine /mol sugar), respectively. Notably, on fructose, strain RE2/pCAK311 produced 1.9- and 2.2-fold more L-lysine (46.7 mM, 0.280 mol L-lysine /mol sugar) than strains ATCC 13032/pCAK311 and Δ gapB/pCAK311, respectively; on sucrose, it produced 1.4- and 2.0-fold more (43.9 mM, 0.250 mol L-lysine /mol sugar).

Evaluation for L-lysine production on isogenic backgrounds. Although strain RE2 was shown to be a high potential host for L-lysine production, there is a possibility that the increased production of L-lysine by strain RE2/pCAK311 may be due to the unknown mutation(s) existing in the strain. As a way to clarify this, we attempted to compare the differences in L-lysine production abilities between GapN and GapA on isogenic backgrounds. For this purpose, the *gapN* gene of strain RE2 was again replaced with the original *gapA* gene to yield strain RE2A. We confirmed the replacement both by sequencing and by enzyme assays (data not shown). The two isogenic strains, RE2 and RE2A, were subject to evaluation for L-lysine production. As shown in Fig. 4, the replacement was found to reduce L-lysine production by strain RE2/pCAK311 to the control levels. On each carbon source, strain RE2A/pCAK311 exhibited the comparable level of L-lysine with strain Δ gapB/pCAK311 (Fig. 4): it produced 23.3 mM (0.140 mol L-lysine /mol sugar), 20.6 mM (0.124 mol L-lysine /mol sugar), and 21.5 mM (0.122 mol L-lysine /mol sugar) L-lysine on glucose, fructose, and sucrose, respectively. These data indicates that the increased L-lysine production by strain RE2/pCAK311 is

attributed to the GapN, namely, a NADPH-generating glycolytic pathway.

DISCUSSION

In this study, replacement of the native NAD-dependent GAPDH with the NADP-dependent GapN from *S. mutans*, followed by isolation of a suppressor mutant with improved growth, led to considerably increased production of L-lysine in *C. glutamicum*. This not only reconfirms the importance of NADPH supply in efficient L-lysine production but also demonstrates that our approach allows reconstruction of a functional glycolytic pathway with a new route of NADPH supply in *C. glutamicum*. Since carbon of sugar inevitably passes through the reaction of the GapN even if it flows *via* glycolysis or *via* the PPP, the engineered strain can generate more NADPH than the wild-type strain, which is clearly reflected by the results of L-lysine production. Considering that 1 mol of L-lysine requires 4 mol of NADPH for its biosynthesis and also that 2 mol of NADPH are inevitably generated at the step of GapN from 1 mol of glucose, the engineered strain can theoretically supply NADPH required for L-lysine biosynthesis up to the yield of 0.5 mol L-lysine/mol sugar, irrespective of the flux distribution between glycolysis and the PPP. Since the yield by strain RE2/pCAK311 (~0.28 mol/mol) is still below the theoretical upper limit, the engineered strain is supposed to supply a sufficient amount of NADPH to its L-lysine biosynthesis exclusively through the reconstructed glycolytic pathway, and therefore involvement of the PPP in NADPH supply may be insignificant in the engineered strain.

Our approach increased the L-lysine yield by 70% on glucose, 120% on fructose, and 100% on sucrose, respectively. The higher gain on fructose than on glucose seems reasonable, because in *C. glutamicum* with GapA, the limitation of NADPH for L-lysine production is known to become more serious when fructose is used, due to a less flux through the PPP on fructose than on glucose (14). This means that the introduction of GapN, which allows sufficient NADPH supply irrespective of the flux distribution between glycolysis and the PPP, can overcome such the sugar-dependent problem. It should be also noted that the gains are generally higher than those obtained by the already established approach to increased supply of NADPH through a redirection of carbon from glycolysis into the PPP (2, 3, 20, 25). The dilemma for the previous approach is that supplying carbon through the PPP is less advantageous than supplying it *via* the glycolytic pathway in terms of carbon yield because the former pathway inevitably involves the release of 1 mol of carbon dioxide accompanied by the oxidation of 1 mol of hexose. Our strategy for supplying NADPH during glycolysis can solve this dilemma, and thus seems to have an advantage for the improvement of amino

acid production.

Our strategy to express the NADP-dependent GapN from *S. mutans* instead of the native NAD-dependent GAPDH can provide an additional source of NADPH during glycolysis, thus facilitating NADPH-dependent pathways. Similar strategies have resulted in increases in NADPH-dependent product synthesis in two other organisms. An increase in ethanol production from D-xylose by *Saccharomyces cerevisiae* has been shown by overexpression of a fungal NADP-dependent GAPDH (34). Martínez et al. (16) also showed improvement in lycopene and ϵ -caprolactone synthesis by overexpression of a *Clostridium acetobutylicum* NADP-dependent GAPDH in *Escherichia coli*. These previous work utilized NADP-dependent GAPDHs that accept both NADP and NAD, and in this sense, our system utilizing the exclusively NADP-dependent GapN from *S. mutans* is assumed to have more directly verified the impact of this metabolic engineering approach. The GapN enzyme from *S. mutans* is irreversible and primarily responsible for the generation of NADPH in *S. mutans* having an incomplete PPP lacking glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (6). Such a key role of the GapN in *S. mutans* assures that the enzyme meets our purpose of creating a *C. glutamicum* strain with increased NADPH availability for effective L-lysine production.

While the growth level of strain GPN was significantly retarded, strain RE2, a spontaneous suppressor mutant originating from strain GPN, exhibited highly improved growth on glucose (Fig. 2 and 3). Based on the fact that we observed no change in either the activity or basic properties of GapN between these two strains, the improvement in growth is presumed to arise from a specific mutation which occurred in another genomic region. However, it is not likely that other mutated enzyme(s) functionally substituting for the GapN reaction emerged in strain RE2, because no similar suppressor mutant was obtained from strain Δ gapAB. Therefore, it is valid to assume that the glycolysis of strain RE2 still fully depends on GapN, as does strain GPN.

The growth retardation observed in strain GPN was first suspected to be due to an imbalance in reducing power caused by excess production of NADPH during glycolysis. In the *E. coli* phosphoglucose isomerase mutant, where extensive carbon flux through the PPP disturbs the cellular reducing power balance and reduces the specific growth rate, the growth impairment is significantly recovered by overexpression of a gene encoding soluble transhydrogenase that oxidizes NADPH to NADP⁺ (5) and partially recovered by the introduction of an NADPH-consuming pathway, such as the poly(3-hydroxybutyrate) synthetic pathway (12). If the same reason is applied to the growth impairment, the recombinant strain, GPN/pCAK311,

which is forced to overproduce L-lysine accompanying the reoxidation of NADPH to NADP⁺, might have been expected to result in improved growth even at low level. However, no such growth restoration was observed in the recombinant strain, suggesting that the growth retardation of strain GPN does not result from the imbalance in NADPH.

Although the growth rate of suppressor mutant RE2 was not sufficiently improved, there is no doubt that the mutation supports cell growth and draws the potential of NADPH-generating GapN, which leads to a significant effect on the L-lysine yield on all carbon sources tested (Fig. 4). Elucidation of the suppressor mutation carried by the strain will give a valuable hint as to further improvement, and thus is under investigation.

ACKNOWLEDGMENTS

We thank Dr. A. Ozaki for encouraging support of our work, and also Drs. S. Hashimoto and S. Koizumi for their useful discussions. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (no. 18580071 and no. 21580089).

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FIGURE LEGENDS

FIG. 1. Influence of deletion of the *gap* genes on sugar utilization of *C. glutamicum*. All strains were grown on BY (top) and MM agar plates supplemented with glucose or acetate (bottom) for 3 days at 30°C. Glc, glucose; Ace, acetate.

FIG. 2. Growth of *gapN*-expressing strain. Wild type *C. glutamicum* (●), strain Δ gapB (■), strain Δ gapAB (◆), strain GPN (▲), and a suppressor mutant from strain GPN (Δ) were cultivated in 5 mL of MM with glucose as a sole carbon source. All cultivations were carried out at 30°C in L-type test tubes with monod shaking at 48 strokes/min. Values are means of replicated cultures, which showed <5% differences between each other.

FIG. 3. Growth of *gapN*-expressing strain, and generation of a spontaneous mutant from the recombinant strain. Wild type *C. glutamicum* (●), strain Δ gapB (■), strain Δ gapAB (◆), strain GPN (▲), and suppressor mutant RE2 from strain GPN (Δ) were cultivated in 5 mL of glucose-based LFG1 medium at 30°C in test tubes with reciprocal shaking at 120 strokes/min. Values are means of replicated cultures, which showed <5% differences between each other. In the case of strain GPN, the lag phase varied from 5 to 6 days in triplicate experiments.

FIG. 4. L-Lysine production and growth levels of *C. glutamicum* strains on various sugars. Wild type *C. glutamicum*, strain Δ gapB, strain GPN, suppressor mutant RE2, and strain RE2A were cultivated in 5 mL of LFG1 medium supplemented with 3% glucose, fructose, and sucrose at 30°C with reciprocal shaking at 120 strokes/min for 4 days. Values are means of replicated cultures, which showed <5% differences between each other. The standard deviations from the means are indicated as error bars. White column, L-lysine concentration on glucose; gray column, on fructose; dotted column, on sucrose; growth (■).

TABLE 1 Sequences of primers used in this study

Primer	Sequence ^a
gapAup5	5'-TAGT <u>GATCA</u> AGTCGAACTCCGGCAAACC-3'
gapAN3	5'-AGCTGGCAGGTGTAGCCCCATGCAACTACCTCGAG ATCGT-3'
gapAC5	5'-ACGATCTCGAGGTAGTTGCATGGGGCTACACCTGC CAGCT-3'
gapAdn3	5'-TGAT <u>GATCA</u> ATCTCGGTCTCTACCAGTCC-3'
gapBup5	5'-CAAT <u>GATCAC</u> ATCTTTACGTAAGGTGGCG-3'
gapBN3	5'-GACCTGGTTGGAGTAGCCGAACTTTGCGGACCACG ATGGCGC-3'
gapBC5	5'-GCGCCATCGTGGTCCGCAAAGTTCGGCTACTCCAA CCAGGTC-3'
gapBdn3	5'- TGAT <u>GATCA</u> AGTTGTTGGCCTGGGAAATC-3'
CGLgapAupXBF	5'-ATTCTCGAGGATCCGATCGCGTCAATGTT-3'
gapNKob3	5'-CGCCATTGACATAATTTTTATATTGTTTTGTCAAGT TGTGTCTCCTCTAAAGATTGTAG-3'
gapNKob5	5'-CTGTAAATCCGTCGTATTTGATATCAAATAATTAG TTCACATCGCTAACGTGGGC-3'
CGLgapAdown900R	5'-ACCGACGATGATCTTGTCAGCCTT-3'
gapNKobF	5'-CTACAATCTTTAGAGGAGACACAACCTTGACAAAAC AATATAAAAATTATGTCAATGGCG-3'
gapNKobR	5'-GCCCACGTTAGCGATGTGAACTAATTATTTGATATC AAATACGACGGATTTAACAG-3'
CGLgapAup900F	5'-TTTGGATCCGTCAATGTTTCGTGATGC-3'
reLysC311F3	5'-GTGGGATCCTTAGGGAGCCATCTTTTGGGGT -3'
reLysC311R3	5'-AGGGGATCCTTGTCGGAAGGGTTCACCTCA -3'

^aBclI sites are underlined and BamHI sites are italicized.

TABLE 2 Specific activities of GAPDH and GapN in recombinant *C. glutamicum* strains^a

Strain	GADH activity (mU/mg)		GapN activity (mU/mg)	
	NAD ⁺	NADP ⁺	NAD ⁺	NADP ⁺
ATCC 13032	439.7±19.3	tr ^b	- ^c	-
ΔgapB	430.1±21.2	-	-	-
GPN	-	n.d. ^d	7.6±4.2	139.9±17.2
RE2	-	n.d.	4.9±0.8	131.9±14.9

^aData represent mean values and corresponding standard deviations using soluble fraction from three independent cultures.

^btr, trace amount (~1.0 mU/mg).

^c-, not detected.

^dn.d., not determined.

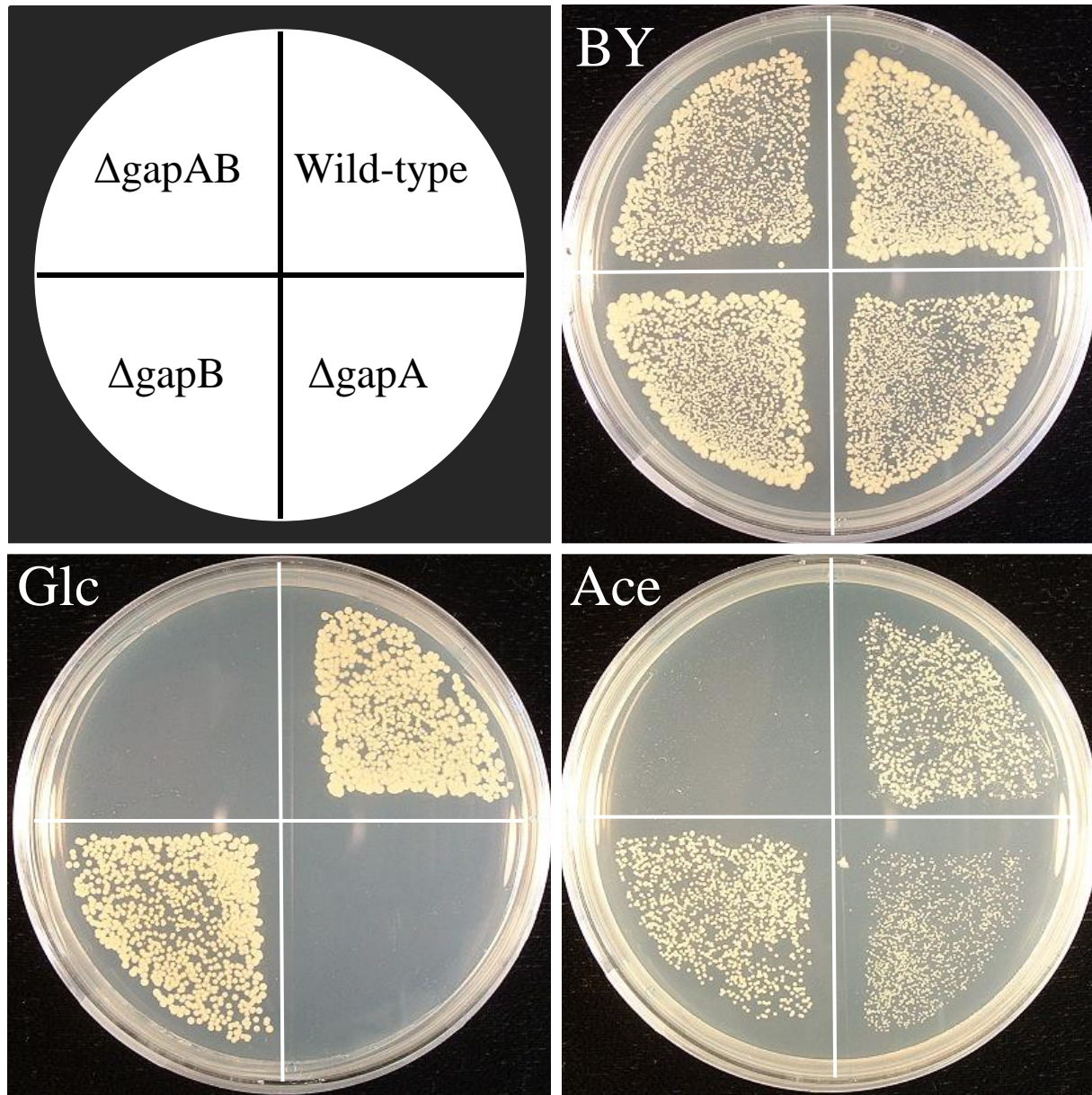


FIG. 1 Seiki TAKENO

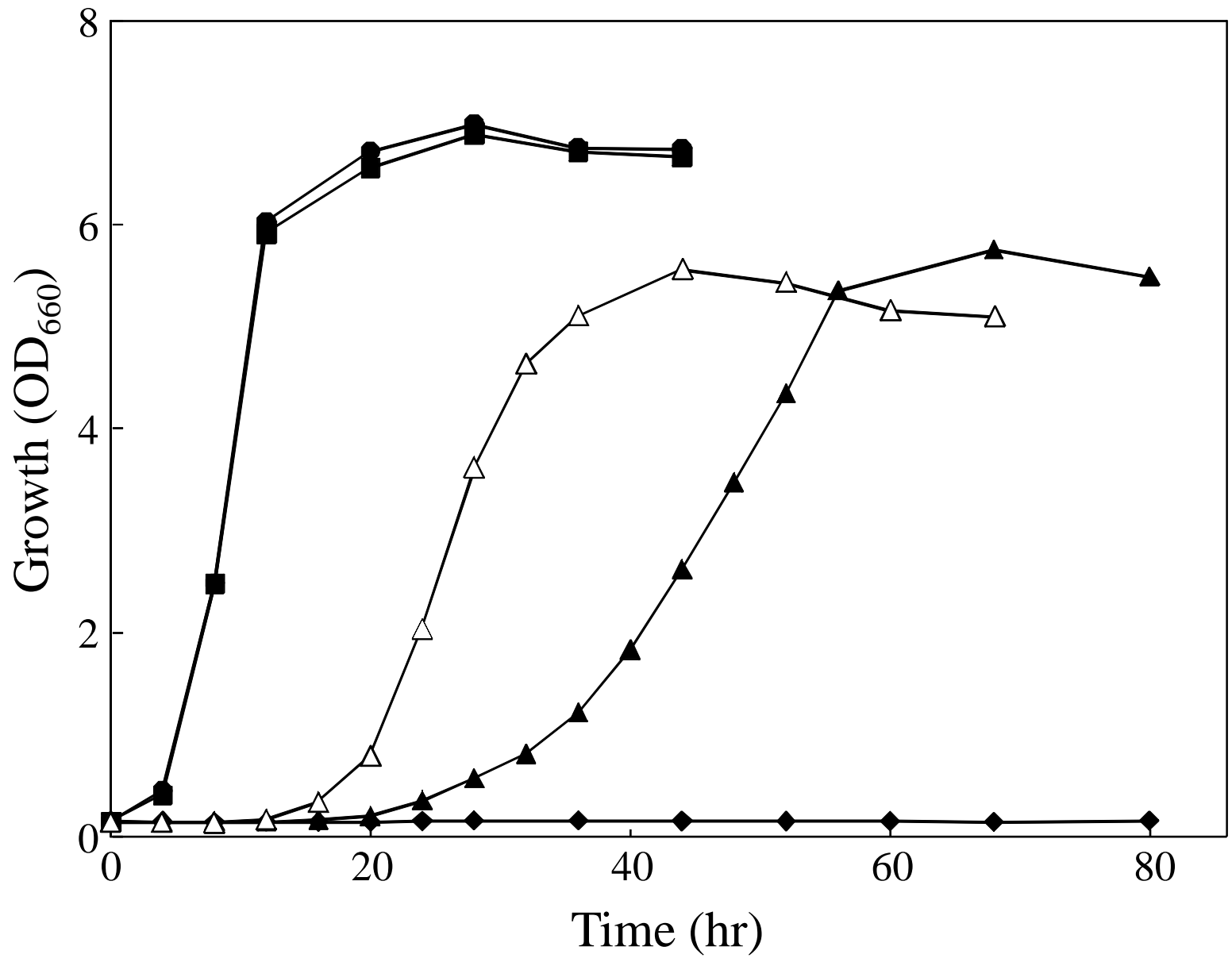


FIG. 2. Seiki TAKENO

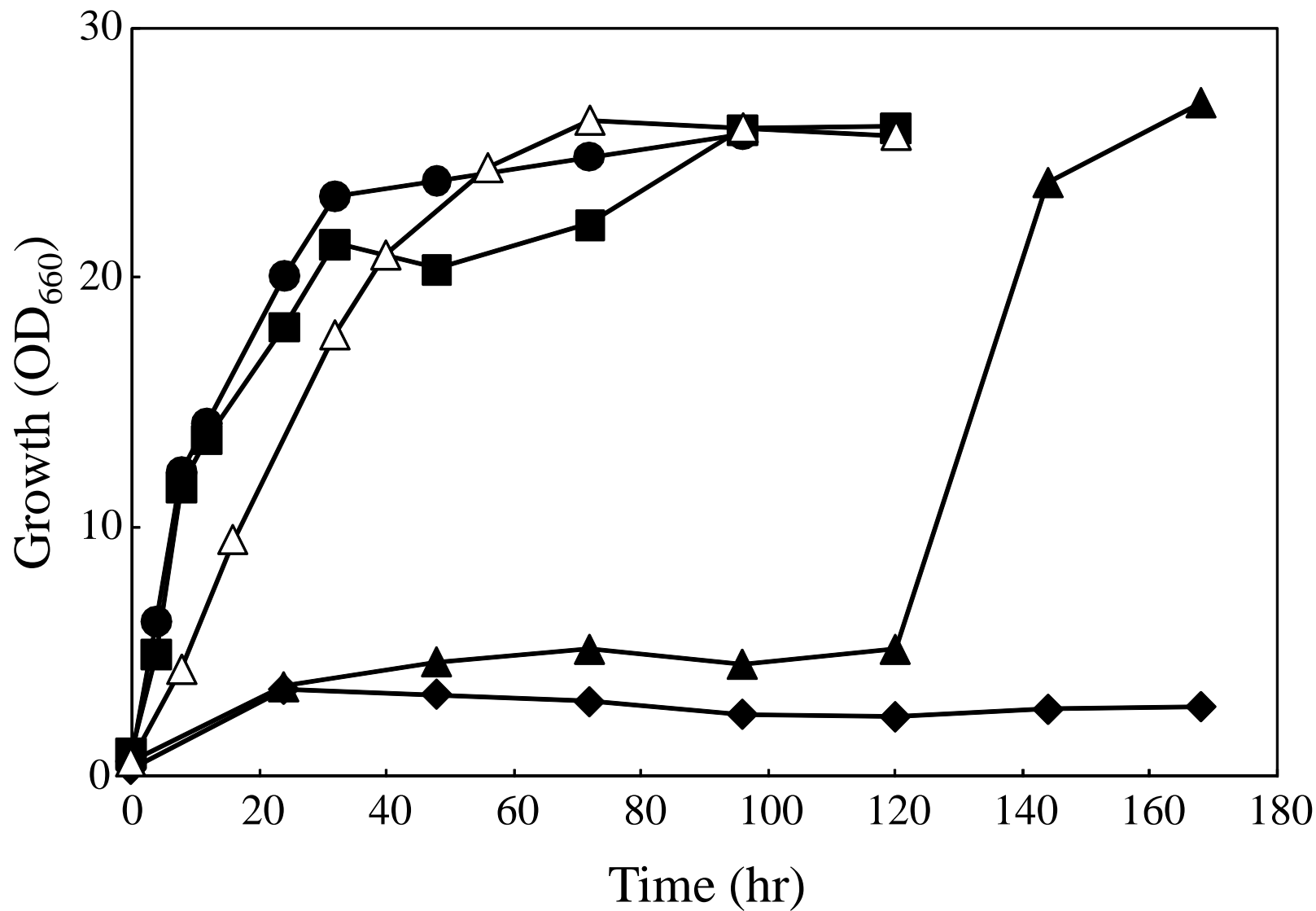


FIG. 3. Seiki TAKENO

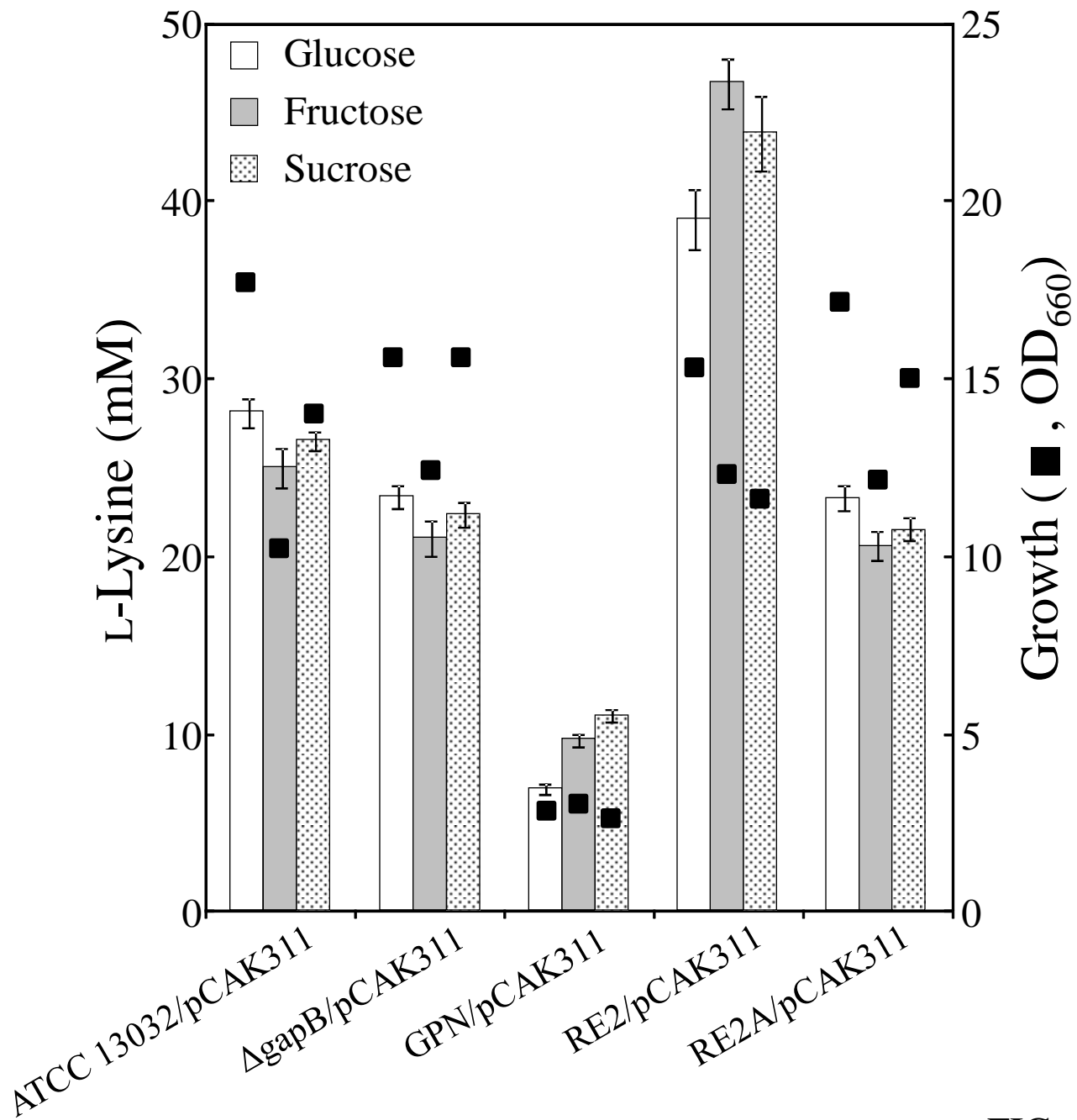


FIG. 4. Seiki TAKENO